EXHIBIT B

Histamine a vasoactive agent with vascular disrupting potential improves tumor response by enhancing local drug delivery

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Running title:

Vasoactive agent Histamine improves drug delivery.

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ABSTRACT

Background: TNF-based ILP is an approved and registered treatment for sarcomas in Europe since 1998 with limb salvage indexes of 76%. We have previously shown the potential of Histamine (Hi) combined to melphalan as an alternative to TNF, with a 66% overall response rate including 33% complete remissions. The aim of this study was to explore the vascular effects of Hi on tumor associated vasculature and evaluate whether the synergistic antitumor effect would still be present with the combination of Histamine and doxorubicin (doxo), an important agent in solid tumor treatment. **Methods**: We used our well-established rat ILP model for in vivo studies looking at tumor response, drug distribution and effects on vessels through fluorescent staining, CD-31 and Perl's method. In vitro studies explored possible drug interactions at cellular level on tumor cells (BN-175) and endothelial cells (HUVEC). **Results**: There was a 17% partial response and a 50% arrest on tumor growth when Hi was combined to doxo, without important side effects, against 100% progressive disease with doxo alone and 29% arrest in tumor growth for Hi alone. Histology documented increased doxo leakage in tumor tissue combined to destruction of tumor associated vasculature, when Hi was added to the ILP. In vitro no synergy between the drugs was observed. **Conclusions**: Hi is a vasoactive drug, targeting TAV and synergizes with different chemotherapeutic agents. These findings further back up the potential translation of Histamine to the clinic opening new frontiers on ILP and organ perfusion.

INTRODUCTION

TNF-based Isolated limb perfusion (ILP) is an approved and registered treatment for sarcomas in Europe since 1998 and is currently carried out in approximately 30 cancer centers with referral programs for limb salvage around the continent[1]. ILP with TNFα and melphalan also yields excellent antitumor effects against melanoma[2] and various other tumors in the clinical setting[3-5]. The mechanism of action is based on the vasoactive effects of TNFα, leading to a significant enhancement of tumor selective melphalan uptake[6] and, secondarily to a complete destruction of tumor vasculature[7].

An important drawback of the use of TNF is its highly toxic nature mandating strict monitoring of leakage to the systemic compartment during ILP. Moreover, this toxic profile of TNF limits expansion of its use to less controllable sites. Therefore, other possible vaso-active drugs were sought and tested in our preclinical rat ILP model as potential candidates[8,9]. In this perspective we showed strong synergy of Histamine (Hi), an inflammatory mediator, when combined to melphalan in ILP, including a 66% overall response rate (OR) with 33% complete responses (CR)[8].

The aim of this study is to evaluate the effects of Hi on TAV by means of histological studies and also explore whether the synergistic effect of Hi would also apply to the combination with doxorubicin (doxo), an important chemotherapeutic drug in solid tumor treatment. Using the experimental ILP model in rats bearing syngenic soft tissue sarcomas, the ability of the combined

treatment to improve tumor response is evaluated. The effects of Hi on endothelial cells and TAV as well as on drug distribution are evaluated *in vivo*, taking advantage of the natural fluorescence of doxo and combining different histological stainings.

MATERIALS AND METHODS

Isolated limb perfusion protocol.

Male inbred Brown Norway rats were obtained from Harlan-CPB (Austerlitz, the Netherlands), weighing 250-300g and were fed a standard laboratory diet *ad libitum* (Hope Farms Woerden, the Netherlands).

Small fragments (3mm) of the syngeneic BN-175 sarcoma were inserted subcutaneously in the right hind leg of the animals as previously described[10]. Tumor growth was measured daily with a caliper and the volume was calculated using the formula $0.4(A^2xB)$ (where B represents the largest tumor diameter and A is the diameter perpendicular to it). When tumor diameter exceeded 25 mm or at the end of the experiment rats were killed by cervical dislocation, under anesthesia.

The treatment consisted of the experimental ILP, previously described[10]. Briefly, 7-10 days after inserting tumor fragments they reached a diameter between 12-15 mm and were amenable to the procedure. Under anesthesia (intraperitoneal ketamine and intramuscular hypnomidate), the inguinal vessels were reached

through an incision parallel to the inguinal ligament, canulated and connected via a roller pump to an oxygenated reservoir where drugs were added in boluses. A groin tourniquet occluded collateral vessels, warranting a proper isolation of the limb.

The 5 mL total volume perfusate consisted of: haemaccel alone (Boehring Pharma, Amsterdam, the Netherlands); haemaccel plus 400 μg doxo (80 μg/mL) (Adriablastina®, Farmitalia Carlo Erba, Brussels, Belgium); haemaccel plus 1000 μg Hi (200 μg/mL) (kindly provided by Maxim Pharmaceuticals Inc., San Diego, CA) or haemaccel, 400 μg doxo and 1000 μg of Hi. For some histological studies perfusate consisted of haemaccel plus 40 μg melphalan (Alkeran Wellcome, Beckeham, UK); or haemaccel, plus 1000 μg Hi (200 μg/mL) and 40 μg melphalan.

Tumor dimensions were measured every day for volume calculation. Response was classified as: progressive disease (PD) increase of more than 25%; no change (NC) volume kept in the range of –25% to +25%; partial remission (PR) decrease between –25% and –99% or complete response (CR), no palpable tumor, initial volume as compared to volume on day nine.

Limb function was clinically observed as the ability to walk and stand on the perfused limb after ILP. On a scale from 0 to 2, grade 0 is a severely impaired function where the rat drags its hind limb; grade 1, a slightly impaired function (can not use it in a normal way, but stand on it); finally grade 2 is an intact function (normal walking and standing pattern).

The studies were done in accordance with protocols approved by the Animal Care Committee of the Erasmus University Rotterdam, the Netherlands

Histologic evaluation after Hi-based ILP.

Two animals for each group were killed by cervical dislocation directly and 24 hours after ILP, tumors and a piece of underlying muscle were excised, fixed in 4% formaldehyde solution and embedded in paraffin. The slides were stained with hematoxylin and eosin and CD-31 by the Pathology department of the Erasmus MC. Images were taken on a Leica DM-RXA microscope supplied with a Sony 3CCD DXC camera.

Perls Iron staining - histologic evaluation after Hi-based ILP.

Two animals for each group were killed by cervical dislocation seven days after ILP, tumors and a piece of muscle from the limb were excised, fixed in 4% formaldehyde solution and embedded in paraffin. The slides were stained by Perl's method. Images were taken on a Leica DM-RXA microscope supplied with a Sony 3CCD DXC camera.

Doxorubicin distribution and TAV evaluation by FITC- lectin ILP

Due to the vaso-active nature of Hi previously described and also seen in TNF-based ILP, we expected an increased accumulation of doxo in tumor tissue with a reduction in the muscular one. To gain insight in the drug distribution in tumoral and normal tissue, four animals for doxo alone and four for Hi plus doxo were submitted to standard ILP plus the addition of 20 μL of FITC-lectin (Sigma) to the perfusate. Directly after the procedure the animals were killed by cervical dislocation, tumors and part of the underlying muscle were excised, snap frozen in liquid nitrogen and stored at –80°C. Thick sections of 25 μm were mounted with Mowiol and evaluated under a confocal microscope. Images were taken with a Sony 3CCD DXC camera.

Cytotoxicity assay.

Direct interaction between doxo and Hi was evaluated in vitro on BN175 tumor cells and endothelial cells.

BN-175 tumor cells (isolated from the spontaneous, rapidly growing and metastasizing soft tissue sarcoma)[11] were grown in RPMI-1640 essential medium (Life Technologies, the Netherlands) supplemented with 10% fetal calf serum and 0.1% penicillin-streptomycin (Life Technologies, the Netherlands).

Cells were plated 24 hours before treatment in 96-wells, flat-bottomed, microtiter plates (Costar, Cambridge, MA, USA) at 10^5 cells per well (100 μL) and allowed

to grow as a monolayer. Next, they were incubated at 37°C in 5% CO_2 for 48 hours in the presence of medium alone or medium plus different concentrations of doxo and Hi. Hi ranged from 0 to 200 μg/mL and doxo from 0 to 5 μg/mL.

Growth of tumor cells was measured using the Sulphorhodamine-B (SRB) assay[12]. In brief, cells were washed with phosphate buffered saline, incubated with 10% trichloric acetic acid for one hour at a temperature of ~4°C and washed again. Cells were stained with SRB for about 15 to 30 minutes, washed with 1% acetic acid and allowed to dry. Protein-bound SRB was dissolved in TRIS (10mM, pH 9.4). Extinction was measured at 540 nm and the percentage of growth inhibition was calculated according to the formula: percentage of tumor cell growth = (test well/control well) x 100%. The drug concentration leading to 50% reduction in absorbance, as compared to control (IC_{50}), was determined from the growth curve. The experiments were repeated four times.

Human umbilical vein endothelial cells (HUVEC) were prepared by collagenase treatment of freshly obtained human umbilical veins and cultured in Human endothelial – SFM/RPMI medium (Biotechnologies, the Netherlands) supplemented with 10% heat inactivated human serum (Biowhitaker, the Netherlands), 20% new born calf serum, human EGF, human vFGF and 0.1% penicillin-streptomycin (Life Technologies, the Netherlands).

HUVEC were plated 24 hours before treatment at $6x10^4$ cells per well and cultured for 48 hours with Hi, in concentrations ranging from 0 to 200 μg/mL and doxo from 0 to 0.5 μg/mL. The growth and IC_{50} were determined in the same way as for the tumor cells.

Statistical analysis.

Kruskal-Wallis and Mann-Whitney U tests were used to evaluate statistical significance of the results. All statistical tests were two-sided and P values less than 0.05 were considered as statistical significant. Calculations were performed on a personal computer using Prism v3.0 software (GraphPad Software Inc.) and SPSS v10.0 for Windows 2000.

RESULTS

Tumor response after ILP.

While tumors grew exponentially in all rats submitted to either control or doxo alone ILP, Hi alone could arrest tumor growth for four days in 2 out of 7 animals (29%). As expected, the best response was seen with the combination of Hi and doxo showing a partial regression in 2 animals (33%) and arrest of tumor growth for approximately six days in 3 animals (50%) ($P<0.01$ on day 8 for Hi plus doxo as compared to Sham; $P = 0.027$ on day 8 for Hi plus doxo as compared to doxo alone). (Figure1).

As previously seen in Hi plus melphalan ILP, Hi either alone or combined with doxo did not inflict systemic side effects. As for regional toxicity only some edema after Hi ILP, both with and without doxo, was observed leading to a temporary grade 1 toxicity in two rats for each group. ILP with doxo alone also caused a

temporary regional toxicity in two rats scoring a grade 1 function with limb edema lasting for 3 to 4 days.

Histology.

Immediately after ILP with Hi plus doxo vasodilation was observed, accompanied by tumoral endothelial cell damage and hemorrhage. Next to that some edema in the tumor was observed. Sham or doxo alone ILP had no effect on vasodilation or hemorrhage and predominantly intact tumoral cells and few necrotic spots were seen. CD-31 stainings corroborate the observation of TAV endothelial cell lining destruction on Hi treated tumors. (Figure 2).

Perls Iron staining - histologic evaluation after Hi-based ILP

In agreement with HE and CD-31 findings, Perl's method documented a hemorrhagic effect linked to Hi administration. Hi treated tumors had iron deposits seven days after ILP, mainly on tumor tissue. Muscle tissue showed few foci but much smaller. Sham and chemotherapeutic drug alone (in this case melphalan) had no iron deposits. These findings further support the specific TAV-targeting action of Hi. (Fig. 3)

Doxorubicin distribution and TAV evaluation by FITC- lectin ILP.

Taking advantage of the natural red fluoresce of doxo we evaluated drug distribution within tumor and muscle by confocal microscopy of thick slides. When ILP was performed with doxo alone some extravasation was observed around perfused (lectin-positive) vessels. Increased extravasation of doxo was seen around tumor vessels, when Hi was co-administered, while in muscle no major effects were noted. Moreover, some areas of doxo leakage could be observed in the tumor, with diffuse or even absent lectin staining, indicating severe damage to the endothelial lining of the tumor vasculature. These observations indicate an increased leakage specifically from the tumor vascular bed when Hi was added to the ILP. (Figure 4)

Direct cytotoxicity of histamine.

To evaluate the potential synergistic action between doxo and Hi in vitro cytotoxicity assays were done on BN-175 tumor cells and on HUVEC. As shown in Figure 5, both agents were capable of killing endothelial cells with an IC_{50} of 200 µg/mL for Hi and an IC_{50} of 0.1 µg/mL for doxo. As for Bn-175 tumor cells, while doxo effectively killed them with an IC_{50} of 0.08 µg/mL, hardly any effect of Hi was noticed with an IC_{50} as high as 500 µg/mL. Combining doxo and Hi in vitro had just an additive effect.

DISCUSSION

In this study we showed the vascular disrupting effect of Histamine. The natural red fluorescence of doxo was combined to vessels labeling by green fluorescent lectin administered in vivo, during ILP. Thus, it was possible to document both the tumor associated vasculature destruction and the better tumor drug distribution when Hi was administered. Additional histological stainings such as CD-31 and Perl's method further documented Hi related endothelial lining disruption and tumor hemorrhage, respectively. It's of note that these effects were more intense on tumor than muscle tissue, which is in agreement with previous findings of a four time increase in melphalan uptake by tumor as compared to muscle[8]. The direct hemorrhagic effect of Histamine, mainly on tumor vasculature is an advantage against the standard drug in use today, TNF-α where this is a secondary effect, seen only around 06 hours after ILP.

No major effects on normal vasculature (e.g in muscle) were seen, indicating a potentially safe profile of HI in terms of side effects and damage to normal tissues. Indeed no serious side effects were observed, only temporary limb edema, completely reversible after 03 to 04 days linked to the use of Hi. It is also noteworthy that no systemic side effects were seen.

Furthermore, in this study we also showed that the anti-tumor effect of Histamine in a regional therapy model, was not restricted to melphalan but it was also present in combination with doxo, another chemotherapeutic agent resulting in tumor regressions or tumor growth arrest in 67% of the rats. Hi alone arrested

tumor growth in 29% of the animals, whilst tumor progression was observed in virtually all rats in sham or doxo alone groups.

Tumor response rates in this study were not as good as those previously reported for the combination of Hi plus melphalan. Yet, doxo alone was also less active than melphalan alone in our ILP model, with no antitumor effect (100% progressive disease) for the first, against 17% partial response and 17% tumor growth arrest for the second[8]. These findings are in accordance with the literature where melphalan is described as the drug of choice for ILP in most centers worldwide with the best response rates and lower complication indexes[10,13]. A possible explanation for the reduced efficacy of doxo could be its cycle dependency, while melphalan does not have this restriction. Taking into account that during an ILP drugs are delivered in a higher dosage but only once, differently from systemic chemotherapy where drugs are given repeatedly, this difference in activity might have played an important role.

Besides the above mentioned, the Hi batch used in this study was also less active both on tumor and endothelial cells (HUVEC) than the previous one, meaning a partial lost on the direct effect of Hi against tumor and endothelial cells. Oddly enough, tumor vasculature targeting with endothelial lining destruction and hemorrhage remained similar in vivo after Hi-based ILP, an observation well documented by the different histologic stainings used. Nevertheless, there certainly was an impact on response rates as Hi alone ILP showed a decrease from the previously reported indexes of 50% to 29% rate in tumor growth arrest. All together, the milder activity of doxo in the regional setting

combined to a weaker Hi probably was responsible for the observed poorer response rates.

TNF-based ILP is an approved and registered treatment for sarcomas in Europe since 1998 and is currently carried out in approximately 30 cancer centers with referral programs for limb salvage around the continent[1]. This success demands further extrapolation to organ perfusions and systemic approach; however, the strong dose limiting toxicity seen with the use of TNF impairs its broader use. We showed here that Hi was also capable of augmenting tumor response when combined to chemotherapeutic drugs. The target of both vasoactive drugs, TNF-α and Hi is the TAV, which explains its beneficial effects on different types of tumors, as long as they have a newly well-developed vasculature. Taking the very short circulation time of Hi and the limited toxicity profile into account Hi has potentially a broad application for the treatment of different tumor types and stands up as an alternative for TNF-α.

In conclusion, the inflammatory mediator Hi acts as a vasoactive drug, targeting the tumor-associated vasculature and is capable of synergizing with different chemotherapeutic agents. These findings support a potential role of Hi in regional treatment and organ perfusions opening new frontiers for further development of these treatment modalities in the clinic, as an important tool in the surgical oncology field.

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LEGENDS:

Figure 1: Tumor response after Histamine-based ILP. Mean tumor volumes +/- SEM are depicted. * P<0.01 on day 8 for Hi+doxo as compared to Sham; P = 0.027 on day 8 for Hi+doxo as compared to doxo alone

Figure 2: Pictures of representative tumor histology right after ILP (HE and CD-31) 16x magnification.

Figure 3: Pictures of representative tumor histology 07 days after ILP stained by Perl's method. 16x magnification.

Figure 4: Pictures of representative tumor histology on thick slides under confocal microscopy right after ILP.

Figure 5: In vitro cytotoxicity of Hi and doxo after 72h incubation with different concentrations. A) BN-175; B) HUVEC. Each point represents an average of four readings. Error bars show standard deviation values

Response curve IHP